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Short communication

Secreted and immunogenic proteins produced by the honeybee bacterial pathogen, *Paenibacillus larvae*

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ABSTRACT

American Foulbrood is a severe disease affecting larvae of honeybee *Apis mellifera*, causing significant decrease in the honeybee population, beekeeping industries and agricultural production. In spite of its importance, little is known about the virulence factors secreted by *Paenibacillus larvae* during larval infection. The aim of the present work was to perform a first approach to the identification and characterization of *P. larvae* secretome. *P. larvae* secreted proteins were analyzed by SDS-PAGE and identified by MALDI-TOF. Protein toxicity was evaluated using an experimental model based on feeding of *A. mellifera* larvae and immunogenicity was evaluated by Western blot, using an antiserum raised against cells and spores of *P. larvae*. Ten different proteins were identified among *P. larvae* secreted proteins, including proteins involved in transcription, metabolism, translation, cell envelope, transport, protein folding, degradation of polysaccharides and motility. Although most of these proteins are cytosolic, many of them have been previously detected in the extracellular medium of different *Bacillus* spp. cultures and have been related to virulence. The secreted proteins resulted highly toxic and immunogenic when larvae were exposed using an experimental model. This is the first description of proteins secreted by the honeybee pathogen *P. larvae*. This information may be relevant for the elucidation of bacterial pathogenesis mechanisms.

1. Introduction

American Foulbrood is a worldwide distributed disease that affects larvae of the honeybee *Apis mellifera*, causing a significant decrease in honeybee populations and productivity (Hansen and Brødsgaard, 1999). The causative agent is *Paenibacillus larvae*, a gram-positive and spore-forming bacterium (Genersch et al., 2006).

Larvae are infected by swallowing food contaminated with spores. These spores germinate in the larval midgut, vegetative cells proliferate, move to the haemocoel and spread causing septicemia. As larvae die their tissues decay and sporulation of bacterial vegetative cells occurs (Hansen and Brødsgaard, 1999).

So far the only described virulence factors of *P. larvae* are metalloproteases (Dancer and Chantawannakul, 1997; Hrabak and Martinek, 2007). It has been proposed that metalloproteases are involved in the inhibition of immune system through the degradation of antibacterial polypeptides (Casteels et al., 1989) and, in the late steps of infection, in the degradation of larval tissues (Katznelson and Lochhead, 1947; Glinski and Jarosz, 1998).

Bacterial secreted proteins perform several important functions, such as provision of nutrients, cell-to-cell communication, detoxification of the environment, and killing of potential competitors. Moreover, secreted proteins of pathogenic bacteria seem to play critical roles in virulence. The aim of the present work was to perform a first approach to identify *P. larvae* secreted proteins and to

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evaluate their immunogenicity as an initial step to detect potential virulence factors.

2. Methods

2.1. Bacterial strains and culture media

P. larvae strains 44 and 29, belonging to the collection of the Department of Microbiology, IIBCE, and isolated from honey of infected beehives located in Durazno and Tacuarembó provinces, respectively, were used in the present work. Isolates were routinely grown on J medium (Hornitzky and Nicholls, 1993).

2.2. Extraction of P. larvae secreted proteins

A *P. larvae* suspension with a cell density equivalent to the 1 value of the Mc Farland scale (10^8 cfu/ml) was prepared in phosphate-buffered saline (PBS). Twenty milliliters of J medium were inoculated with 1 ml of the bacterial suspension, incubated at 37 °C with shaking for 72 h, centrifuged at 14,000 × *g* for 20 min and supernatant was lyophilized. The lyophilized product was suspended in 1/20 of its original volume in PBS.

2.3. SDS-PAGE

Twenty microliters of *P. larvae* secreted proteins (approximately 40 μg) were electrophoresed in a polyacrylamide gel electrophoresis under denaturing conditions, as described by Laemmli (1970) and stained with 0.1% (v/v) Coomassie brilliant blue. Wide Range protein molecular weight marker (BioRad, 6500–20,0000 kDa) was used.

2.4. MALDI-TOF

Proteins were excised from the SDS-PAGE and analyzed by MALDI-TOF MS and MS/MS at the CEQUIBIEM, Buenos Aires, Argentina. Peptide mass fingerprints were analyzed using the Mascot Daemon software package (Matrix Science, Boston, MA). The search parameters were: maximum of one missed cleavage by trypsin, charged state of +1, and mass tolerance of less than 60 ppm or ±0.5 Da. Protein excision, SDS-PAGE and MALDI-TOF identifications were performed by duplicate.

The identified proteins were analyzed by SignalP, SecretomeP, PSORT, TMHMM, and PROSITE to characterize their predicted mode of secretion, cellular localization, and protein domains. SignalP and SecretomeP were used to evaluate if a protein is secreted by the classical or non-classical Sec pathway, respectively (Bendtsen et al., 2004; Nielsen et al., 1997), PSORT was used to predict protein localization (Nakai and Horton, 1999), TMHMM to predict transmembrane helices (Krogh et al., 2001) and PROSITE was used to bring together general information about proteins (Hulo et al., 2006).

2.5. Antiserum preparation

Animal experiments were conducted in accordance with procedures authorized by IIBCE, Montevideo, Uruguay. Female CD-1 6–8-week-old mice from the breeding facilities at IIBCE were used and provided with food pellets and tap water *ad libitum*. Antiserum was raised in the mouse using a four-dose immunization schedule. All doses contained a mixture of *P. larvae* vegetative cells and spores. The first dose was administered in Freund's complete adjuvant. Subsequent doses were given at 2-weekly intervals in Freund's incomplete adjuvant and the serum was collected 1 week after the last immunization by submandibular vein puncture.

2.6. Western blot

P. larvae secreted proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Towbin et al., 1979). Western immunoblots were performed using a 1:100 dilution in PBS/Tween 20/1% skim milk of mouse polyclonal antiserum raised against *P. larvae*.

2.7. Toxicity bioassays

Worker larvae from the progeny of a single wild-mated honeybee queen (*Apis mellifera ligustica*) maintained in a disease-free apiary at the USDA Bee Research Lab (Beltsville, MD, USA) were used for this study. Larvae were collected and transferred to plastic microtiter trays (96 wells) for rearing (Evans, 2004). Larvae were fed *ad libitum* with an excess of a liquid diet consisting of 66% royal jelly, 6% glucose, 6% fructose and 1% yeast extract in sterile distilled water. Larval food was supplemented with *P. larvae* secreted proteins at final concentrations of 5, 50 and 500 ng/μl. Groups of 12 larvae were used in each case. Secreted proteins from *P. larvae* isolates 44 and 29 were used. Larvae were fed with contaminated larval diet for the first 48 h after grafting and thereafter, normal larval diet was used for feeding. Control larvae were fed with normal larval diet throughout the experiment. Plates were incubated at 34.5 °C with high humidity for several days. Each day, larvae were taken out from the incubator and examined. Larvae were classified as dead when they lost their body elasticity or displayed a color change to brownish. The number of dead larvae was recorded, and surviving larvae were transferred to new wells filled with fresh food.

3. Results

3.1. Identification of P. larvae secreted proteases by MALDI-TOF

Ten different proteins ranging between 35 and 136 kDa were identified among the *P. larvae* secreted proteins (Fig. 1 and Table 1). Only two proteins were predicted to harbor the cleavable N-terminal signal peptides indicating its secretion via the classical Sec pathway, and three proteins were predicted for secretion by the alternative Sec pathway. Accordingly, PSORT predicted that two of these proteins were situated in the extracellular medium and TMHMM algorithm predicted they have one transmembrane helix.

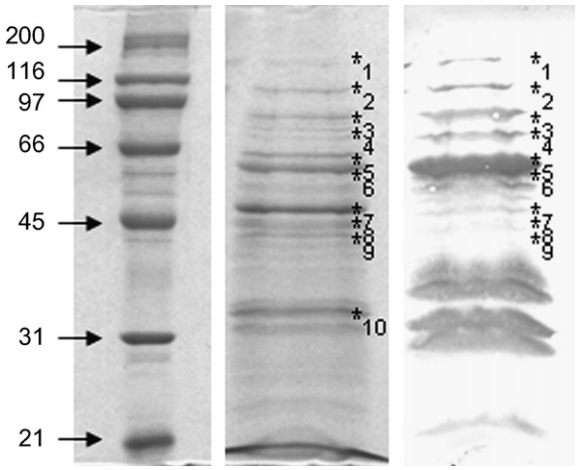

Fig. 1. 1: Wide range protein molecular weight marker (BioRad). 2: SDS-PAGE analysis of *P. larvae* secreted proteins. 3: Western blot analysis of *P. larvae* secreted proteins using antiserum raised against vegetative cells and spores of *P. larvae*. Asterisks indicate the identified proteins.

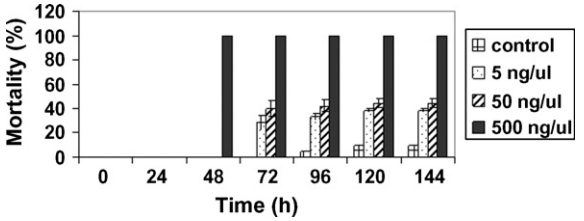

Fig. 2. Toxicity of *P. larvae* secreted proteins. Cumulative mortality percentage of *A. mellifera* larvae fed with larval food supplemented with *P. larvae* secreted proteins at final concentrations of 5, 50 and 500 ng/μl, and control larvae ($n = 12$).

3.2. Toxicity and immunogenicity of P. larvae secreted proteins

To evaluate the toxicity of *P. larvae* secreted proteins during infection, these proteins were administered to *A. mellifera* larvae within the food. *In vivo* assays were performed using 5, 50 and 500 ng/μl of secreted proteins. The experiment was performed for 7 days, period in which the mortality percentage of control group remained under 12.5%. *P. larvae* secreted proteins were toxic for larvae and mortality percentage increased with protein concentration and incubation time (Fig. 2). Western blots analysis of *P. larvae* secreted proteins using antiserum generated against *P. larvae* vegetative cells and spores, indicated that most of the secreted proteins are immunogenic (Fig. 1). However, the immune dominant antigens could not be identified, since they were not detected by SDS-PAGE.

4. Discussion

In the present manuscript we present a preliminary identification of the secretome of *P. larvae*. Ten different proteins involved in transcription, metabolism, cell envelope, transport, protein folding and motility were identified. Most of them have been previously found in the extracellular medium of different bacteria (Antelmann

Table 1
List of *P. larvae* secreted proteins identified by MALDI-TOF.

Protein mass	Access number	Protein name	Conserved domains (PROSITE)	Function category	Localization (PSORT)	Secretion (SignalP)	Secretion (SecretomeP)	Transmembrane domains (TMHMM)
136 kDa	gi|167461256	DNA-directed RNA polymerase subunit beta	No	Transcription	Cytoplasm	No	No	0
98 kDa	gi|167464089	Aconitate hydratase 1	Aconitase family signature 1 and 2	Metabolism	Cytoplasm	No	No	0
77 kDa	gi|167465343	Translation elongation factor G	GTP-binding elongation factors signature	Translation	Cytoplasm	No	No	0
67 kDa	gi|167462593	S-layer domain protein	Fibronectin type-III domain profile	Cell envelope	Outside	Yes	Yes	1
60 kDa	gi|167461076	ABC-type transport systems, periplasmic components	Bacterial extracellular solute-binding proteins, family 5 signature	Transport	Cytoplasm	No	Yes	0
57 kDa	gi|167463819	Chaperonin GroEL	Chaperonins cpn60 signature	Protein folding	Cytoplasm	No	No	0
50 kDa	gi|197743423	Glutathione reductase	Pyridine nucleotide-disulphide oxidoreductases class-I active site	Metabolism	Membrane	No	No	0
49 kDa	gi|167464406	Chitin binding protein	Fibronectin type-III domain profile	Degradation of polysaccharides	Outside	Yes	Yes	1
46 kDa	gi|167461759	Enolase (2-phosphoglycerate dehydratase)	Enolase signature	Metabolism	Cytoplasm	No	No	0
39 kDa	gi|167464311	Flagellin	No	Motility	Cytoplasm	No	No	0

et al., 2005; Chitlaru et al., 2006, 2007; Gohar et al., 2005; Walz et al., 2007). Although these proteins were detected in the secretome, most of them were classified as cytoplasmic, lacking recognizable signal sequences. This may be because some secretion signals do not conform to the consensus sequence. However, the possibility of contamination of culture supernatants with cytosolic proteins cannot be ruled out.

To further characterize *P. larvae* secreted proteins and to evaluate their role in pathogenicity we used an experimental model using *A. mellifera* larvae. Toxicity assays demonstrated that *P. larvae* secreted proteins are toxic for honeybee larvae. Although in controlled experiments ten spores are enough to cause disease, the infective doses in the field are not known (Woodrow, 1942), making difficult to estimate the amount of *P. larvae* secreted proteins during field infection. In order to cover different situations, a 1000-fold range of amounts of proteins was used. Nevertheless, the lowest dose used (5 ng/μl) resulted toxic.

Among the proteins secreted by *P. larvae* many of them are strongly related to virulence of other pathogenic microorganisms. Chaperonin GroEL is produced by prokaryotic and eukaryotic cells to assist in the correct folding of proteins (Henderson and Jensen, 2006). However, in pathogenic bacteria this protein can also act as intracellular, cell surface, or extracellular signal in the course of infection, as reported for *Chlamydia trachomatis*; *Chlamydia pneumoniae* and *Salmonella enterica* (Buchmeier and Heffron, 1990; Henderson and Jensen, 2006; Sanchez-Campillo et al., 1999) between others.

S-layer proteins have also be related to virulence, being potentially involved in the resistance to host immune response in the case of *Campylobacter fetus* or promoting interactions with host cells in the case of *Bacillus cereus* (Pei and Blaser, 1990; Kotiranta et al., 1998).

Enolase is another interesting example. Although it is a cytoplasmic enzyme involved in glycolytic pathways, several authors found that bacterial enolases are expressed on the cell surface or even secreted to the medium, as described for *Bacillus anthracis*, *B. cereus* and *Bacillus thuringiensis* (Delvecchio et al., 2006; Lamonica et al., 2005). Moreover, these authors found that enolase is an immunogenic protein, coinciding with our results. Enolases have been reported as plasminogen activators; they bind to the infected host plasminogen activating its proteolytic activity and allowing the bacteria to acquire surface-associated proteolytic activity, constituting an advantage for tissue invasion (Agarwal et al., 2008; Bergmann et al., 2001; Esgleas et al., 2008; Pancholi and Fischetti, 1998; Sha et al., 2009).

Lastly, flagellin has also been involved in the pathogenicity of *B. thuringiensis* (Zhang et al., 1993).

Although no metalloproteases were identified between the secreted proteins, several studies have demonstrated their secretion by *P. larvae* (Antúnez et al., 2009; Dancer and Chantawannakul, 1997; Hrabak and Martinek, 2007).

The present article is a first approach to elucidate the secretome of *P. larvae*. Our results suggest that *P. larvae* secretes a large repertoire of toxic and immunogenic proteins which may be essential for its virulence and the course of larval infection.

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